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Inactivation of natural microflora and inoculated *Listeria innocua* on whole raw shrimp by ozonated water, antimicrobial coatings, and cryogenic freezing

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ABSTRACT

Shrimp have been associated with outbreaks of foodborne illnesses. A survey was conducted to determine the microbiological safety and quality of frozen whole raw shrimp, purchased at markets in the Northeast United States. Shrimp were composed of 32 brands from 9 different countries of origin. The average aerobic, psychrotrophic, coliform, and *Enterobacteriaceae* microbial counts were 3.64, 4.06, 0.02 and 1.43 log CFU/g, respectively. Of shrimp samples that were *Listeria* spp. positive (21.9%), 12.5% were identified as *Listeria monocytogenes*. No *Salmonella* spp., *Escherichia coli* O157:H7, *Vibrio* spp., or *Staphylococcus aureus* were detected. The survival of natural microflora and inoculated *Listeria innocua* on shrimp were investigated after treatments with ozonated water, antimicrobial coating and cryogenic freezing, alone or in combination. Ozonated washes, cryogenic freezing and antimicrobial coating treatments, applied singly, reduced the natural bacteria or *L. innocua* by <2 log CFU/g; however, in combination, treatments provided additive or synergistic reductions of the natural bacteria and *L. innocua*. The chitosan coating with allyl isothiocyanate, in combination with cryogenic freezing, inactivated more than 5 log of *L. innocua* and natural microorganisms. These information may be valuable for seafood processors and distributors to adopt intervention strategies to enhance the safety and extend the microbial shelf-life of shrimp.

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1. Introduction

According to Centers for Disease Control and Prevention (CDC), from 2005 to 2010, 39 outbreaks and 2348 illnesses were linked to imported food from 15 countries. Of those outbreaks, seafood (17 outbreaks) was the most common source of implicated imported foodborne disease outbreaks, and nearly 45 percent of the imported foods causing outbreaks came from Asia (CDC, 2012).

The United States imported *ca.* 1.27 billion pounds of shrimp in 2011, at a value of $5.16 billion (USDA, 2012). Additionally, there was *ca.* 3 million pounds of shrimp produced domestically in 2010 (Texas Aquaculture Association [TAA], 2012). The per capita consumption of shrimp in the U.S. is estimated to be 4.1 lbs. *Listeria monocytogenes* has been identified as a pathogen of concern for shrimp, based on product detentions and recalls (Ababouch, Gandini, & Ryder, 2005; Wan Norhana, Poole, Deeth, & Dykes, 2010). Contamination of seafood processing plants and the seafood itself is problematic as the incidence of *Listeria* contamination can be high, and seafood is often consumed raw (Chen, Pyla, Kim, Silva, & Jung, 2010; Gurtler & Kornacki, 2007, chap. 17; Pagadala et al., 2012). Most shrimp imported into the U.S. comes from developing countries, where an appropriate HACCP system may not be applied. In addition, according to the U.S. Government Accountability Office (GAO), only about 2% of imported seafood is inspected (Gilbert, 2011).

Nonthermal processing and chemical preservation are used to treat heat-sensitive food to avoid quality loss. Shrimp is one such food when the product is sold raw. Ozone in both gaseous and dissolved-aqueous forms was reported to inactivate microorganisms on foods, including fungi, yeast, parasites and viruses (Guzel-

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Seydim, Greene, & Seydim, 2004), and bacteria, such as *L. monocytogenes* (Paranjpye, Peterson, Poysky, & Eklund, 2008) and the tetracycline-resistant *Listeria innocua* (Vaz-Velho, Silva, Pessoa, & Gibbs, 2006). However, there have been conflicting results, as reported by Kim, Silva, Chamul, and Chen (2000) and Crapo, Himelbloom, Vitt, and Pedersen (2004), that ozone washing or spraying treatments had very limited antimicrobial efficacy for seafood.

Cryogenic freezing, which directly exposes food to subfreezing temperatures, has been used for the preservation of shrimp quality. Boonsumrej, Chaiwanichsiri, Tantratian, Suzuki, and Takai (2007) reported that cryogenic freezing caused less quality changes of tiger shrimp than air-blast freezing; and Lopkulkiaert, Prapatsornwattana, and Rungsardthong (2009) also found that cryogenic freezing combined with sodium bicarbonate significantly improved yield, freezing time, freezing rate, cutting force and color of white shrimp, when compared with other freezing methods. Recently, edible antimicrobial coatings and packaging have been developed and several applications for meat, poultry, and seafood have been reviewed (Campos, Gerschenson, & Flores, 2011; Gennadios, Hanna, & Kurth, 1997). Some studies showed that the antimicrobial coating alone or when combined with other techniques, such as gamma irradiation and modified atmosphere packaging, could inactivate or retard microbial growth and prolong the shelf-life of shrimp (Jiang, Liu, & Wang, 2011; Mastromatteo, Danza, Conte, Muratore, & Del Nobile, 2010; Ouattara, Sabato, & Lacroix, 2001). However, there is very limited information regarding the combination of these treatments; therefore, there is a need to develop nonthermal technologies, which can reduce microbial contamination and levels of pathogens on shrimp.

The objectives of this study were to: 1) conduct a survey to assess microbiological safety and quality of shrimp from local markets, including the incidence of *Listeria*, and 2) evaluate the effect of ozone washing, antimicrobial coating, and cryogenic freezing, when applied alone or in combination on the survival of background microflora and inoculated *L. innocua* on shrimp surfaces.

2. Materials and methods

2.1. Background microflora and foodborne pathogens

The U.S. Food and Drug Administration (FDA) standard methods described in the FDA's Bacteriological Analytical Manual (BAM) were used (FDA, 2012). Approximately 375 g of frozen raw shrimp were removed from 5 lb boxes, and blended (Waring Laboratory, Torrington, CT) with buffered peptone water (BBL-Difco Laboratories, Sparks, MD) at a 1:10 dilution for 2 min. One mL of diluted shrimp sample was then pipetted onto aerobic plate count (APC), coliform and *Enterobacteriaceae* Petrifilms™ (3M, St. Paul, MN, USA) in duplicate. The films were incubated according to the manufacturer's specifications. In addition, a second set of APC films were inoculated and incubated at 22 °C to obtain psychrotrophic counts. A BAX Polymerase Chain Reaction (PCR) test was then conducted using the BAX® System Q7 (Dupont Qualicon, Wilmington, DE, USA) to determine the presence of *Listeria* spp., *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Vibrio* spp. If a positive PCR result was obtained, FDA's approved methods were used to recover viable foodborne pathogens (FDA, 2012). The *Listeria* species that were recovered (Table 2) were identified using API test strips (BioMeroiux, Marcy l'Etoile, France).

2.2. Antimicrobials

Chitosan (Low Molecular Weight, 150 kDa, 75–85% deacetylation) and allyl isothiocyanate (AIT) (95% purity) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetic acid, lactic acid and levulinic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). Lauric arginate ester (LAE) solution (CytoGuard®) containing 20% LAE was obtained from A&B Ingredients (Fairfield, NJ).

2.3. Sample preparation for coating treatment

After thawing overnight in a refrigerator (4 °C), shrimp were divided into three groups: the first group was used to test background microflora in shrimp without temperature abuse, the second group was used to test background microflora in shrimp at an abused temperature (room temperature [22 °C] for 12 h) before any treatments, and the third group was inoculated with *L. innocua*.

2.4. Inoculum and inoculation

Three *L. innocua* strains (ATCC 51742, 33090, and 33091) obtained from the American Type Culture Collection (Manassas, Va., U.S.A.) were reported to have similar inactivation kinetics as pathogenic *L. monocytogenes* used in previously reported studies for both thermal and nonthermal processing technologies (Sommers, Geveke, & Fan, 2008; Sommers, Kozempel, Fan, & Radewonuk, 2002; Sommers, Scullen, & Sites, 2010). Each strain was maintained on Tryptic soy agar (BD-Difco) at 0–2 °C. Prior to experiments, each strain was cultured independently in 30 mL Tryptic soy broth (BBL/Difco) on a rotary shaker (150 RPM) at 37 °C for 18 h. The aliquot (30 mL) of fresh culture from each strain was combined. This inoculum (90 mL) was used to inoculate shrimp samples. The concentration of the inoculum was *ca.* 9.0–9.5 log CFU/mL.

The prepared inoculum (90 mL) was added to 5 L of sterile deionized water mixed with 200 pieces of shrimp, and kept in a refrigerator (4 °C) overnight to allow bacterial attachment. The inoculums were then decanted and the shrimp were allowed to drip dry, before being placed in a sterile polypropylene pan. The population of *Listeria* on the shrimp after inoculation was *ca.* 8.2 log CFU/g.

2.5. Antimicrobial treatment

2.5.1. Ozone wash treatment

An aluminum alloy basket, 15.2 × 15.2 × 15.2 cm ($L \times W \times H$), with perforated openings of 0.8 cm × 2.5 cm ($L \times H$) was specifically constructed for this study. Shrimp were placed in the basket and manually agitated, while 9.5 L/min of ozonated water, generated on site with OSW-3 ozonated water generator (Ozone Solutions, Inc., Hull, IA, USA), at 20 kPa with a temperature of 19–22 °C and an ozone concentration of 1.6–1.9 ppm, was immediately sprayed directly on the shrimp for 55–65 s, using a size 20 nozzle spray head (Loc-Line #51845, Lake Oswego, OR).

2.5.2. Coating solution preparation and coating treatment of shrimp

Three coating solutions were prepared for this study. Chitosan coating solution #1 (CC1) included 200 mg chitosan in 10 mL of an acid solution, containing 2% each of acetic, lactic and levulinic acids. Chitosan coating solution #2 (CC2) included chitosan coating solution #1 (CC1) + 6% (v/v) AIT; and Chitosan coating solution #3 (CC3) included chitosan coating solution #1 (CC1) + 5% (v/v) LAE. Each mixture was stirred with a magnetic stir bar on a stir plate until the polymer was completely dissolved. Shrimp were dipped in coating solutions for 1 min. The non-coated (control) and coated shrimp were placed in a bio-safety cabinet to dry at room temperature for 2 h, prior to other treatments or microbial analysis.

2.5.3. Cryogenic freezing treatments

Shrimp were placed in a perforated basket (40 × 40 × 15 cm [$L \times W \times H$]) built in Cryo-Test Chamber (Air Products and

Chemicals, Allentown, PA, USA), which exposed the shrimp to liquid nitrogen vapor in a controlled manner. The shrimp were cryogenically frozen (−75 °C) for 2 min. Frozen shrimp samples (five shrimp, *ca.* 21 g each) were then placed in individual poly-nylon bags (Uline, Inc., Philadelphia, PA), and kept frozen at −20 °C for 4 days prior to microbiological analysis.

2.5.4. Binary and tertiary treatment combinations

Additionally, ten antimicrobial treatments in binary and ternary combination were used to inactivate naturally-occurring bacterial and inoculated *L. innocua* on shrimp. Each treatment and corresponding code used in this study are listed in Table 1.

2.6. Microbial analysis

The frozen-treated and control shrimp were transferred into individual sterile stomacher bags with 100 mL of 0.1% peptone water, and hand-messaged for 1 min. Five shrimp per treatment were used. Ten mL of the shrimp sample was then pipetted into each sterile test tube, and serial dilutions of the resultant bacterial suspensions were prepared with 0.1% peptone water. For background microflora, 1 mL of diluted sample was placed on Petrifilm™ (3M, St. Paul, MN, USA) following the procedures provided by the manufacturer, and incubated for 48 h at 22 °C (psychrotrophic bacteria) or 24 h at 37 °C (aerobic plate counts). For *L. innocua*, 0.1 mL of the diluted samples were surfaced-plated in duplicate onto PALCAM agar plates (Difco) with Palcam selective supplement (Oxoid, England) and incubated at 37 °C for 48 h.

2.7. Statistical analysis

Data from three independent experiments (duplicate samples at each sampling time), for a total of six data points per treatment ($n = 6$), were analyzed using Analysis of Variance with SAS version 9.1 software (SAS Institute, Carry, NC). Duncan's multiple range test was used to determine the significant differences of mean values. Significance was defined at $p < 0.05$.

3. Results

3.1. Survey of bacteriological quality of raw whole shrimp

Thirty-two frozen whole raw shrimp samples, acquired from local retail markets, representing nine countries of origin, were tested for their bacteriological quality. The results are shown in Table 2. Populations of aerobic mesophilic bacteria (APC) ranged from 2.77 to 5.39 log CFU/g with an average of 3.64 log CFU/g. Psychrotrophic bacteria were 2.60–6.36 with an average of 4.06 log CFU/g. Coliforms were found in only one sample, with 0.7 log CFU/g. *Enterobacter* spp. ranged from zero (below the detection limit) to 3.69, with an average of 1.43 log CFU/g. No *Salmonella* spp., *S. aureus*, or *E. coli* O157:H7 was detected by PCR. Although two of the 32 samples were positive by PCR for *Vibrio* spp., no viable *Vibrio* were isolated using a variety of methods, which could be due to the susceptibility of *Vibrio* spp. to sub-freezing temperatures (Januario & Dykes, 2005; Vasudevan, Marek, Daigle, Hoaglund, & Venkitanaranan, 2002).

Seven of the 32 shrimp samples were found to contain viable *Listeria* spp., which were subsequently identified as *L. monocytogenes*, *Listeria welshimeri*, and *L. innocua*. The focus of the second half of the study, therefore, centered on the inactivation of the *L. monocytogenes* non-pathogenic surrogate *L. innocua*, inoculated onto whole raw shrimp in bio-safety level 1pilot plant setting.

Table 1
Treatment and abbreviation code used in this study to report results.

Treatment	Code
Control (no treatment)	NT
Ozone wash	OW
Chitosan coating	CC1
Chitosan coating with AIT	CC2
Chitosan coating with LAE	CC3
Cryogenic freezing	CF
Ozone wash followed by cryogenic freezing	OW + CF
Ozone wash followed by chitosan coating	OW + CC1
Ozone wash followed by chitosan coating with AIT	OW + CC2
Ozone wash followed by chitosan coating with LAE	OW + CC3
Chitosan coating followed by cryogenic freezing	CC1 + CF
Chitosan coating with AIT followed by cryogenic freezing	CC2 + CF
Chitosan coating with LAE followed by cryogenic freezing	CC3 + CF
Ozone wash followed by chitosan coating and cryogenic freezing	OW + CC1 + CF
Ozone wash followed by chitosan coating with AIT and cryogenic freezing	OW + CC2 + CF
Ozone wash followed by chitosan coating with LAE and cryogenic freezing	OW + CC3 + CF

3.2. Effect of ozone, cryogenic freezing, and antimicrobial coatings on inactivation of natural microorganisms on shrimp

The antimicrobial efficacy of ozone washing, cryogenic freezing, and antimicrobial coatings against natural microflora on shrimp, used singly or in combination, is shown in Figs. 1 and 2. Psychrotrophic counts in untreated shrimp were 5.85 log CFU/g. Ozone washing and cryogenic freezing treatments reduced only 0.89 and 0.61 log CFU/g of psychrotrophic bacteria.

Chitosan coating (CC1) did not significantly reduce the population of background bacteria in shrimp, while adding AIT or LAE to the chitosan coating significantly ($p < 0.05$) enhanced their antimicrobial effectiveness. Chitosan + AIT (CC2) treatment was the most effective in bacterial reduction, achieving a more than 4.55 log CFU/g reduction of background bacteria, followed by the chitosan + LAE (CC3) treatment, which inactivated 2–3 log CFU/g of psychrotrophic bacteria.

The combination of two treatments (the hurdle approach) provided more microbial reduction of background bacteria in shrimp than when they were used alone. The bacterial reduction for most combinations was sum of two treatments (additive effect). However, the combination of freezing treatment along with coating treatments exhibited a synergistic antimicrobial effect, which was significant at $p < 0.05$. For example, CC1 + CF treatments inactivated 2.09 log while CC1 and CF alone only achieved 0.25 and 0.61 log reductions of natural psychrotrophic bacteria, respectively (Fig. 1).

There were three treatments in ternary combinations of ozone, freezing and coatings. All three combined treatments showed additional antimicrobial effects against natural bacteria in shrimp. OW + CC1 + CF reduced natural psychrotrophs by 2.32; OW + CC2 + CF reduced psychrotrophs to undetectable levels (<1.30 log CFU/g); and OW + CC3 + CF reduced psychrotrophs by 2.32 log CFU/g.

Populations of natural bacteria in shrimp stored at abusive temperatures prior to application of interventions significantly increased, while psychotrophic bacteria in control samples were 9.19 log CFU/g (Fig. 2), which is more than 3 log higher than those without temperature abuse.

Ozone washing, cryogenic freezing and coating treatments applied alone or in combination achieved a greater microbial reduction corresponding to each treatment than those treatments that were not temperature-abused. Ozone washing and cryogenic freezing reduced 1.08 and 0.51 log CFU/g of psychrotrophic bacteria.

Table 2
Survey of bacteriological quality of raw whole shrimp.

Sample #	Country of origin	Farmed or wild	Additives as stated on label	APC (log CFU/g)	Psychrotrophs (log CFU/g)	Coliforms	Enterobacter (log CFU/g)	BAX *Listeria* spp.	*Listeria* species[a]
S1	Mexico	Farm	Sodium metabisulfite	3.17	3.34	0	1.9	–	
S2	Ecuador	Farm	None listed	3.14	3.48	0.7	1.48	–	
S3	India	Farm	None listed	3.46	3.56	0	0	–	
S4	Ecuador	Farm	Sodium metabisulfite	3.69	3.73	0	0	+	Lw
S5	Thailand	Farm	Sodium tripolyphosphate	4.03	3.61	0	3.33	–	
S6	Thailand	Farm	Salt & sodium phosphates	3.79	2.6	0	2.5	–	
S7	India	Unknown	Salt & tripolyphosphates	3.8	3.92	0	2.13	–	
S8	India	Farm	None listed	5.39	3.73	0	1.95	–	
S9	USA	Wild	Salt	4.06	5.78	0	2.23	+	Lm and Li
S10	USA	Farm	None listed	4.96	6.36	0	3.89	–	
S11	USA	Wild	None listed	3.56	4.88	0	0	–	
S12	Indonesia	Farm	None listed	3.64	3.89	0	0	–	
S13	USA	Wild	None listed	2.59	3.51	0	0	–	
S14	Thailand	Farm	Salt	3.21	4.51	0	1.8	–	
S15	Bangladesh	Farm	Sodium phosphates & salt	4.12	4.63	0	1.8	+	Lm
S16	Indonesia	Farm	Salt & tripolyphosphate	2.77	4.04	0	1.7	+	Lm and Li
S17	Bangladesh	Farm	Sodium phosphates	4.47	4.74	0	0	–	
S18	Thailand	Farm	Salt	3.24	3.66	0	1.85	–	
S19	Thailand	Farm	Salt	2.77	2.8	0	0	–	
S20	Thailand	Farm	Salt & sodium phosphates	3.55	3.61	0	0	–	
S21	Thailand	Farm		3.3	6.38	0	0.69	–	
S22	Indonesia	Farm		3.97	5.53	0	0	+	Lm
S23	Bangladesh	Farm	Salt & sodium tripolyphosphate	5.12	5.2	0	0	–	
S24	Indonesia	Farm	Salt & sodium tripolyphosphate	3.26	4.03	0	0	–	
S25	Thailand	Farm	None listed	3.03	3.23	0	3.02	–	
S26	Vietnam	Farm	None listed	3.89	3.09	0	2.2	+	NR
S27	Indonesia	Farm	None listed	4.33	4.89	0	2.92	–	
S28	Belize	Farm	None listed	3.06	3.29	0	2.78	–	
S29	Mexico	Wild	None listed	2.96	2.99	0	1.48	+	NR
S30	Thailand	Farm	Salt	3.69	3.52	0	2	–	
S31	Thailand	Farm	Salt & sodium tripolyphosphates	3.77	3.88	0	2.18	–	
S32	Indonesia	Farm	Salt & sodium tripolyphosphates	2.79	3.58	0	1.81	–	
Mean (SEM)				3.64 (±0.12)_	4.06 (±0.17)	0.02 (±0.02)	1.43 (±0.21)	7/32 = 21.9% Positive	4/32 = 12.5% Lm positive

[a] Lm = *L. monocytogenes*; Li = *L. innocua*; Lw = *L. welshimeri*; NR = not recoverable.

The antibacterial effects of 7 treatments in binary combination of coating, ozone, and freezing significantly reduced populations of the native bacteria. Among them, CC2 + CF showed the greatest effective antimicrobial activity, which reduced over 7.33 log CFU/g of psychrotrophs on the shrimp (Fig. 2).

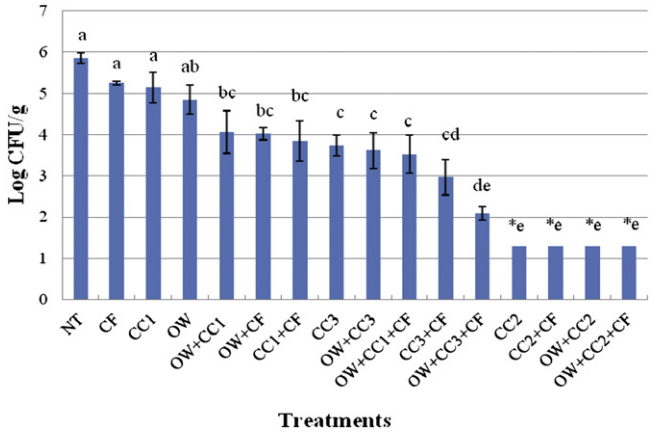

Fig. 1. Survival of natural psychrotrophs on shrimp after treatments. NT: control; CF: cryogenic freezing; OW: ozone wash; CC1: coating 1; CC2: coating 2; CC3: coating 3. Other treatment codes refer to Table 1. Error bars represent the standard deviation of the mean. Data having a common letter are not significantly different ($p > 0.05$). *Under detection limit (<1.30 log CFU/g).

The ternary combination also exhibited additional antibacterial effects. OW + CC1 + CF reduced natural psychrotrophs by 3.58 log CFU/g. OW + CC2 + CF reduced the psychrotrophs to undetectable levels (<1.30 log CFU/g). OT + CT3 + FT reduced psychrotrophs by 3.73 log CFU/g.

Similar reductions by each treatment were observed for mesophilic bacteria (APC) and the brief results are listed in Table 3.

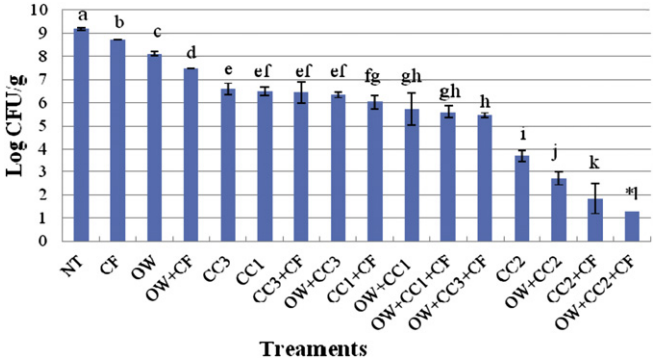

Fig. 2. Survival of natural psychrotrophs on shrimp after treatments. The shrimp samples were placed at 22 °C for 12 h before any treatment. NT: control; CF: cryogenic freezing; OW: ozone wash; CC1: coating 1; CC2: coating 2; CC3: coating 3. Other treatment codes refer to Table 1. Error bars represent the standard deviation of the mean. Data having a common letter are not significantly different ($p > 0.05$). *Under detection limit (<1.30 log CFU/g).

Table 3
Treatments grouped by log reduction of natural bacteria and *L. innocua*.

	<1 log	1–2 log	2–3 log	3–4 log	4–5 log	>5 log
Psychrotrophs	CF	OW OW + CF	CC1 CC3 CC3 + CF OW + CC3	CC1 + CF OW + CC1 OW + CC1 + CF OW + CC3 + CF		CC2 CC2 + CF OW + CC2 OW + CC2 + CF
APC	CF	OW OW + CF	CC1 CC3 CC3 + CF	OW + CC1 OW + CC3	CC2 CC1 + CF OW + CC1 + CF OW + CC3 + CF	CC2 + CF OW + CC2 OW + CC2 + CF
L. innocua	CF	OW OW + CF	CC1	CC3 CC1 + CF OW + CC1	CC2 CC3 + CF OW + CC2 OW + CC3 OW + CC1 + CF OW + CC3 + CF	CC2 + CF OW + CC2 + CF

3.3. Effect of ozone, cryogenic freezing, and antimicrobial coatings on the inactivation of L. innocua on shrimp

The antimicrobial efficacy of ozone, cryogenic freezing, and antimicrobial coatings against *L. innocua* inoculated on shrimp, used alone or in combination, is shown in Fig. 3.

The initial population of *L. innocua* on shrimp was *ca.* 8.18 log CFU/g. Reductions of 1.64 and 0.37 log CFU/g were achieved by ozone and freezing treatments, respectively.

The coating treatments with chitosan (CC1), chitosan + AIT (CC2) and chitosan + LAE (CC3) reduced *L. innocua* by 2.59, 4.48, 3.16 log CFU/g, respectively. Similar to the background bacteria, chitosan + AIT was more effective in inactivating *L. innocua*, followed by chitosan + LAE.

Various binary combinations also had additional or synergistic antimicrobial effects. The significant synergistic effect was observed for the combination of coating treatments followed by cryogenic freezing. For example, CC2 + CF achieved a 5.64 log reduction, which is more than the sum of 4.48 log (CC2) and 0.37 log (CF), when applied singly.

The combination of OW + CC2 + CF exhibited the greatest antibacterial effect against *L. innocua*, which reduced *Listeria* from 8.18 log CFU/g to an undetectable level (<2.30 log CFU/g), a more than 5.88 log reduction, followed by OW + CC3 + CF and OW + CC1 + CF.

4. Discussion

Contamination of seafood processing environments and finished seafood with *Listeria* is a relatively common occurrence and has been well documented for *ca.* 30 years (Buchanan, Stahl, Bencivengo, & Dell Corral, 1989; Chen et al., 2010; Gudmundsdottir, Gudmundsdottir, Einarsson, Kristinsson, & Kristjansson, 2006; Gurtler & Kornacki, 2007, chap. 17; Jinneman, Wekell, & Eklund, 1999; Pagadala et al., 2012; Rahimi, Shakerian, & Raissy, 2012; Valdimarsson, Einarsson, Gudbjornsdottir, & Magnusson, 1998; Weagant et al., 1988). Microbial contamination of shrimp can occur at various stages of production: culture environment, in the processing plant environment, during handling, during transportation on the ship and/or truck, and during storage where fluctuating temperature conditions may occur (Anonymous, 2002). Multiple outbreaks of foodborne illnesses associated with seafood, including shrimp, have been reported (CDC, 2012; Wan Norhana et al., 2010). Class 1 recalls (that could cause serious health problems) have occurred in the United States (Jinneman et al., 1999) and have resulted in severe economic losses to producers in both domestic and international markets (Paranjpye et al., 2008).

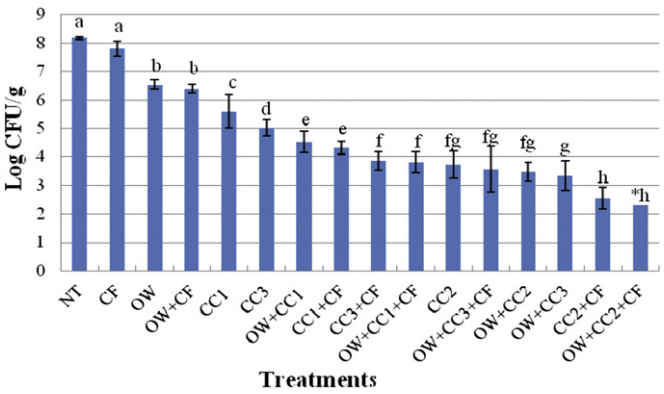

Fig. 3. Survival of *L. innocua* on shrimp after treatments. NT: control; CF: cryogenic freezing; OW: ozone wash; CC1: coating 1; CC2: coating 2; CC3: coating 3. Other treatment codes refer to Table 1. Data having a common letter are not significantly different ($p > 0.05$). *Under detection limit (<2.30 log CFU/g).

In our survey, 21.9% of shrimp samples were found to be positive for *Listeria* spp., and 12.5% were identified as *L. monocytogenes*, which is similar to results obtained in the studies of seafood and seafood processing environments listed above. Two samples were PCR-positive for *Listeria*, which is both freeze- and refrigeration-tolerant, but a viable *Listeria* colony was not recovered from the samples. Although foods containing low levels of *L. monocytogenes* (<10^2 CFU/g) pose very little risk, eliminating the higher concentrations can reduce the number of predicted cases by >99% (Chen, Ross, Scott, & Gombas, 2003). In addition to pathogenic contamination, spoilage microbes also impact the bacteriological quality and shelf-life of shrimp. In our survey of 37 samples from 9 country origins, populations of APC and psychrotrophs averaged 3.64 and 4.06 log CFU/g, respectively (Table 2). The natural occurring bacteria increased 3 log CFU/g after shrimp was held at 22 °C for 12 h when compared to those held at 4 °C (Figs. 1 and 2). The results from the present study, therefore, highlight the importance of appropriate HACCP programs from farm to table and the necessity of additional interventions for the seafood industry to ensure microbiological quality and safety.

The concept of food preservation as hurdle technology was developed by Leistner in the 1970s (Leistner, 1992), which has been used to improve the safety, quality and stability of foods when a single process is not good enough to achieve those goals. Different foods, including fruits and vegetables, bakery, dairy, or fish products have been reported using this concept (Leistner, 2000).

In the present study, ozone washing (OW), cryogenic freezing (CF) and chitosan coating (CC1) treatments reduced <2 log CFU/g of natural bacteria or *Listeria*, when they were applied singly. Crowe, Skonberg, Bushway, and Baxter (2011) reported that aqueous ozone sprays at concentrations up to 1.5 mg/L reduced aerobic

bacterial and *L. innocua* by 0.28 and 1.17 log, respectively. Kim et al. (2000) found minimal log reductions (<1.0 log) of psychrotrophs on catfish immersed in 10 mg/L ozone solutions. Crapo et al. (2004) reported <1 log reduction of *L. innocua* on salmon fillets treated with ozone washes and sprays of up to 1.5 mg/L ozone. Other researchers found that pure chitosan films or coatings only achieved <2 log reduction of *L. monocytogenes*, *E. coli* O157:H7, or *Salmonella* spp. (Anacarso et al., 2011; Chen, Jin, Gurtler, Geveke, & Fan, 2012; Duan, Park, Daeschel, & Zhao, 2007; Zivanovic, Chi, & Draughon, 2005). Therefore, the combination of these treatments is needed when greater levels of inactivation are required.

The reduction of the combined treatment was up to 7.33 log CFU/g (Fig. 2). Among them, the combination of chitosan coating with AIT (CC2) showed the most effectiveness. AIT is a broad-spectrum antimicrobial agent, effective against various microorganisms, Gram-positive and Gram-negative bacteria, alike (Isshiki, Tokuoka, Mori, & Chiba, 1992; Lin, Kim, Du, & Wei, 2000; Rhee, Lee, Dougherty, & Kang, 2003). Numerous researchers have used AIT to retard the growth of pathogens and spoilage microorganisms with concentration as high as 1200–20,000 μg/mL (Gurtler, Rivera, Zhang, & Sommers, 2010; Muthukumarasamy, Han, & Holley, 2003; Winther & Nielsen, 2006). In our previous study, a chitosan coating with 60 μL/mL AIT reduced more than 5 log CFU/cm^2 of *Salmonella* inoculated on surface of whole cantaloupe (Chen et al., 2012), and a polylactic acid coating with 500 μl AIT completely inactivated 7 log CFU/mL of *Salmonella* in liquid egg albumen (Jin & Gurtler, 2011).

LAE is commonly-used commercially to control spoilage and *Listeria* spp. in processed meats. Previous research by Sommers, Mackay, Geveke, Lemmenes, and Pulsfus (2012) and Sommers, Rajkowski, Sheen, Samer, and Bender (2011) indicated that 5% LAE was capable of inactivating multiple foodborne pathogens, including *Listeria* spp., *Salmonella* spp, and *S. aureus*, when sprayed onto processed meats by 2–3 log CFU/g. The results obtained using CC3 are consistent with previously-published results. Interestingly, when processed meats were treated with ultraviolet light or flash pasteurization, prior to application of LAE, inactivation of pathogens increased to more than 5 log CFU/g.

Both AIT and LAE are permitted for use as a food preservative and regarded by the FDA as a "generally recognized as safe" (GRAS) (Delaquis & Mazza, 1995; Isshiki et al., 1992; Kim, Ahn, & Shin, 2002). While LAE is colorless, odorless, and tasteless, use of AIT in food systems is limited because of its pungent odor, which can significantly affect the taste of foods (Chacon, Buffo, & Holley, 2006; Delaquis & Mazza, 1995; Kim et al., 2002). The degree of sensory impact is also concentration-dependent. Chacon et al. (2006) reported that beef samples treated with AIT concentrations lower than 1480 ppm only had a faint residual odor. In the present study, no AIT aroma was noticed on shrimp after the AIT coating treatments were applied and dried overnight. Also, no significant differences in appearance were detected by visual observation among all samples (data not shown). Although chitosan coating with LAE (CC3) was not as effective as chitosan coating with AIT (CC2) within the concentrations tested in this study, LAE could be an advantageous over AIT since LAE doesn't have a pungent odor like AIT. Ultraviolet light and flash Pasteurization, in combination with LAE, a component of the coating solutions, resulted in additive and synergistic reductions of multiple foodborne pathogens (Sommers et al., 2010, 2012) on processed meats. Because coatings, which included AIT and LAE, were effective, future research should investigate the use of coatings containing both AIT and LAE.

Most combinations exhibited additive antimicrobial effects, which is expected. However, interestingly, the combinations of coatings with cryogenic freezing (CC1 + CF, CC2 + CF and CC3 + CF) showed a synergistic effect, *ca.* 1 log greater reduction than the sum of each reduction applied singly. It is well-established that bacterial cells can be injured by adverse environmental stresses such as freezing, and that injured cells subsequently become more sensitive to antimicrobials under these conditions (Jiang, Neetoo, & Chen, 2011; Wesche, Gurtler, Marks, & Ryser, 2009). Ryser and Marth (1989) also reported that freezing induces cryo-injury and thus sensitizes *L. monocytogenes* to the antimicrobial agents.

Table 3 lists the treatments applied in this study, grouped by reduction of natural bacteria and *L. innocua*, which provides displays the overall performance of each treatment or their combinations in microbial reduction. This information may be valuable for seafood processors to choose one treatment, binary or ternary combinations according to their food safety needs. As noted in Fig. 3, OW + CC2 + CF provided the greatest log reductions on shrimp surfaces; however, there are materials and equipment costs associated with each treatment. Additional treatments (ternary or binary combinations) will increases costs more than a single treatment. Among the coating treatments, CC2 and CC3 cost more than CC1, since AIT or LAE is needed. Therefore, Table 3 may also help seafood processors find a balance between microbial reduction and materials and equipment costs. Cost evaluations, shelf-life and sensory effects on shrimp with these applied treatments will be determined in future scaled-up studies, prior to commercialization of this technology.

5. Conclusions

Foodborne illness outbreaks and product quarantines indicate that shrimp can be a food of microbiological safety concern. The populations of APC and psychrotrophs in this study were, on average, 3.64 and 4.06 log CFU/g, respectively, while 21.9% of shrimp samples were *Listeria*-positive and 12.5% were identified as *L. monocytogenes*. Ozone, antimicrobial coatings and cryogenic freezing achieved different degrees of microbial inactivation and their combinations provided additive or synergistic effects against naturally-occurring bacteria and *L. innocua* on shrimp. Among them, chitosan coatings with AIT (CC2) in combination with cryogenic freezing were sufficient to inactivate 5-log CFU/g of *L. innocua* and natural bacteria on shrimp surfaces. The results of the present study highlight the importance of additional interventions in the seafood industry to ensure microbiological safety and shelf-life of raw shrimp. This study demonstrates that hurdle technology is an effective approach to reducing microbial loads on shrimp surfaces to a desirable level by the application of different combinations of treatments, which may provide processors or distributors viable options for designing nonthermal interventions to improve the microbiological safety and quality of shrimp.

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